DETECTION OF TERATOGENIC SUBSTANCES IN ACIDIC MINE WATER SAMPLES USING THE FROG EMBRYO TERATOGENESIS ASSAY-XENOPUS (FETAX)

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PREFACE

The Frog Embryo Teratogenesis Assay-Xenopus (FETAX) has been developed to detect teratogenicity of pure compounds and complex mixtures. The application of this system as a quick screen to determine possible teratogenicity of test substances, allows for prioritization of those substances for further testing in established mammalian systems. FETAX provides data on malformation, mortality and inhibition of growth and development that can be statistically analyzed to determine the teratogenic risk of the test compound.

This study determined the presence of teratogenic agents in acidic mine water samples from the Tar Creek area of Oklahoma. The results obtained were another step in the validation of FETAX as a useful method of determining agents that cause birth defects.

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INTRODUCTION

Short-term screening assays, such as the Ames mutagenicity test[1], have been developed to test potential toxicity, mutagenicity and carcinogenicity of compounds. The development of rapid screening assays for teratogenicity is presently taking place. One such assay involves use of embryos of the South African Clawed Frog, Xenopus laevis. Xenopus has been previously used in a bioassay[2] and the procedure has been recently standardized and its advantages summarized by Dumont and co-workers[3]. They have named this bioassay "FETAX" (Frog Embryo Teratogenesis Assay:Xenopus) and have employed this system to test complex mixtures. FETAX meets the criteria set forth by Kimmel et al.[4] for short-term in vitro teratogenesis assays. This assay was employed in the present study to detect teratogenic risk of Tar Creek water.

Tar Creek is located in northeastern Oklahoma, extending from just north of the Kansas border south to the Neosho River and at the time of this study was ranked as one of the most hazardous sites in the U.S. for toxic metals pollution[5]. The metals, including iron, lead, zinc and cadmium, occur as sulfide ores which are readily oxidized. When the lead and zinc mines were closed, groundwater and surface runoff began filling the extensive underground mine caverns. Since 1979, surface seeps of the mine waters have impacted Tar Creek with low pH and highly metal-contaminated discharge. Heavy metal contamination of water supplies is of great concern in this area and has been implicated as possible causes of cancer and other health problems[5].

In this study, we tested the effect of several concentrations of water samples from four Tar Creek area sites on survival, gross malformation, development, and growth of the Xenopus embryos. Two of the sites sampled were discharges of water directly from the mines. The other two sampling sites were on Tar Creek at points above and below the mine water discharges. We found that water from the upper portion of the creek was neither teratogenic nor toxic and caused only minor growth inhibition in some instances. The mine water discharges were toxic to the embryos and inhibited growth. The samples from the

lower portion of the creek were toxic below the expected pH tolerance of Xenopus embryos but were teratogenic in the tolerable pH range, growth was also inhibited. Divalent cations such as lead, zinc and cadmium were probably responsible for the observed teratogenicity and growth inhibition as sample water passed over the chelating resin Chelex® 100 was no longer teratogenic or growth inhibiting. We have concluded that FETAX provided excellent information for prioritizing the testing of surface waters for toxic and teratogenic agents using established mammalian systems.

EXPERIMENTAL

Description of Sampling Locations

Tar Creek is located in the Eagle-Picher lead and zinc mining field. The latter comprises over 150 km^2 (ca. 60 mi^2) in southeastern Kansas and northeastern Oklahoma. Tar Creek has a drainage basin covering 134.7 km^2 (ca. 52 mi^2) with northernmost headwaters in Kansas. Tar Creek discharges into the Neosho River at Miami, Oklahoma.

Water samples from selected sites on Tar Creek and from mine discharges were collected during this study (Figure 1). Tar and Treece (S18 T29N R23E), the northernmost site, is upstream from known mine discharges. This site is located on the Kansas-Oklahoma border adjacent to extensive tailings piles. Stream flow is intermittent at this site and is primarily from

surface runoff after precipitation. OWRB #4 (S29 T29N R23E) is a mine discharge site that flows periodically into Tar Creek and is south (downstream) of Tar and Treece. Mine discharge from this site is dependent upon the water levels in the mines, the latter being strongly influenced by the amount of precipitation. The Horse Pasture (S7 T28N R23E) is another mine discharge site and is located south (downstream) of OWRB #4. The discharge at this site flows continuously into Tar Creek. Tar and Miami (S30 T28N R23E) is south (downstream) of the Horse Pasture site and is adjacent to Northeastern Oklahoma A and M College. Tar Creek water at this site receives discharge from the previous sites.

Water Sample Collection

Water samples for use in the bioassay and for heavy metal analyses were collected from the Tar Creek sites on four occasions. All surface water samples were collected midway between sediments and the water surface while samples from the mine discharge were collected from the discharge openings. Water samples used in the assay were collected in one-liter polypropylene bottles to overflow to eliminate airspaces and refrigerated at 2-3°C until use. The samples for heavy metal analyses were collected in polypropylene bottles and acidified to a pH<2. All analyses were performed according to standardized

methods[6]. Concurrent with sampling, an assessment of the general water quality was made using an Orion model 211 pH meter for the determination of pH and YSI meters for evaluation of specific conductivity, dissolved oxygen and temperature.

Animal Care and Breeding

The water for holding tanks of Xenopus adults and larvae was filtered through an activated carbon filter (Barnstead™) and aerated for 48 h prior to use. The water was routinely tested for pH, oxygen content, hardness, heavy metal content and total organic carbon[7].

Adult Xenopus were obtained from Xenopus I® (Ann Arbor, MI) and kept in glass aquaria for a minimum of 10 d until use. Adults were fed beef liver and lung obtained from a local packing house. The meat was supplemented with baby vitamins (Polyvisol®). Prior to mating, the female received 1000 I.U. of human chorionic gonadotropin (Sigma®, St. Louis, MO) and the male 500 I.U. injected into the dorsal lymph sac. Amplexus ensued within 2-6 h and egg deposition within 9-15 h from the time of injection.

After breeding, the adults and any fecal material were removed from the tank and the embryos collected by scraping the eggs into 55 mm plastic Petri dishes. The jelly coats of the

embryos were removed by gentle swirling for 3.5-4 min in a 2% w/v cysteine solution which had been prepared using aerated tap water and adjusted to pH 8.1 with NaOH. Although we routinely remove the jelly coat, others do not[3]. The presence of the jelly coat has not been shown to alter toxicity[8].

Normally developing blastulae were initially selected by sorting them from necrotic eggs and abnormally cleaving embryos. A second selection was then performed to ensure that only normal embryos were used in the test.

Water Sample Preparation and Animal Exposure

Due to the low pH of the water at three of the sample sites, the testing of the sample water from each site was divided into two groups: one with no adjustment of the pH (pH unadjusted) and one adjusted to a pH of 7.0 (pH adjusted) using NaOH or acetic acid as appropriate[9]. The unadjusted and adjusted samples were then diluted using aerated tap water to obtain a concentration range from 10-100% sample water. The tap water diluent for the experiments using sample water from the second collection was filtered through a 0.22 μ Nucleopore® filter to remove bacteria and the pH adjusted to a normal tap water pH of 7.8 when necessary. For each sample concentration, two groups of embryos of ten each were placed in covered 55 mm plastic Petri dishes

each containing eight ml of solution. Four groups of ten embryos each were placed in eight ml of aerated tap water as controls. All glassware used in this study was washed in dilute HCl, rinsed, washed in dilute NaOH and then rinsed in distilled water. The solutions were changed after the first 48 h of each experiment with sample concentrations prepared as just described. The changes were performed carefully to prevent damage to the embryos.

Prior to the preparation of the sample concentrations pH, specific conductivity and dissolved oxygen measurements were taken from the sample bottles at both 0 and 48 h to determine the degree of change in the water samples from the time of collection until use.

During sample preparation additional samples for heavy metal analyses were prepared at 0 and 48 h of the experiment to determine the amount of metal oxidation during storage. For the first sample collection at 0 h of the experiment 100 ml of each sample was acidified to a pH<2 and analyzed for heavy metals. An additional 100 ml sample from each sample site was allowed to sit open at 23°C for 48 h before preservation for analysis to simulate the amount of metal oxidation that would take place in sample dishes over a 48 h period. For the final three sets of

sample collections, 50 ml of each sample was preserved for analysis at 0 and 48 h as described above. In addition, a second 50 ml portion of each water sample was preserved at 0 and 48 h after adjustment of the pH to 7 to determine the amount of metal precipitation during titration of the adjusted samples.

The Use of a Chelating Resin to Remove Metal Ions Prior to Toxicity Testing

In order to determine if heavy metal contamination affected embryo survival, growth and development, 700 ml of the fourth collection of Tar and Miami sample water was twice passed over a 20 ml Chelex® 100 (Bio-Rad®) column, to remove divalent cations. To ensure proper development, 30 mg of $CaCl_2$ and 9 mg of $MgCl_2$ were added back to the water. A portion of this water was adjusted to pH 7.0 using NaOH while the remaining portion was left unadjusted at pH 6.4. These samples were then diluted with dechlorinated tap water to obtain a concentration range from 10 to 100% sample water.

The weakly acidic chelating resin Chelex® 100, a styrene divinylbenzene copolymer, binds metal ions using paired iminodiacetate ions as chelating groups[10]. The use of Chelex® 100 for removal of metal ions has been reviewed and provides a rapid method for separating ionic and complexed metals [11].

Therefore, the resin was used to remove metal ions such as lead, cadmium, zinc and iron from the sample water in order to determine if heavy metals were responsible for the toxic, teratogenic, and growth inhibiting effects that were observed.

Data Collection

At 24, 48, 72 and 96h, the dead embryos were removed from each dish and that number recorded. The number of live-malformed embryos and the stage of development according to Nieuwkoop and Faber[12] were determined. Embryos were scored as malformed whenever an alteration of gross morphology was observed. Typically, deformation of cranial, heart, gut and tail structures were seen as well as micropthalmia and edema. Malformed embryos which had died were not included in the number malformed. Death at 24 (stage 27) and 48 h (stage 37) was ascertained by the embryo's skin pigmentation, structural integrity and irritability while at 72 (stage 42) and 96 h (stage 45), the lack of heartbeat in the transparent embryo was an unambiguous sign of death.

At 96 h, the number of dead and malformed embryos was recorded for each concentration. The pH of each sample concentration was taken at this time or at the time 100% mortality occurred in a dish to help determine the effect of pH

on the observed toxicity or teratogenicity. Surviving embryos were fixed in 0.5% formalin and the head-tail length of each embryo was measured using a Radio Shack® digitizer and the data input directly to a Radio Shack® model 16 microcomputer.

Data Analysis

A general polynomial curve fitting routine was used to analyze the data for survival and malformation while the t Test for grouped observations was used to test for a significant reduction in growth. These statistical tests were part of a microcomputer software package[13] adapted by us for the analysis of these experiments. The software can perform all statistical tests and print and plot the results as well as store data relevant to the experiments.

RESULTS

Analysis of the Dilution Water

Our modification of the FETAX system of Dumont et al.[3] was designed to test the teratogenicity of complex mixtures, such as Tar Creek water, as well as pure substances. This testing necessitated the use of dechlorinated tap water in which to rear the Xenopus embryos, larvae and adults. Periodically, samples of the tap water were analyzed for hardness and heavy metal content. Throughout the study, control embryos (40/test) were raised in dechlorinated tap water. The malformation and death rates of the controls averaged less than 5%. These results and the data from the water analysis indicated that the dechlorinated tap water was not significantly teratogenic nor toxic[7].

Dissolved Oxygen Content, pH and Specific Conductivity of the Water Samples

Water quality parameters for temperature, dissolved oxygen, pH and specific conductivity were determined in the field (Table

1). The results from Tar and Treece indicated that oxygen concentrations and pH values were within acceptable limits[14]. Specific conductance was elevated due to increased concentrations especially of calcium and magnesium derived from previous acid impact on the region's carbonate bedrock. The OWRB #4 and Horse Pasture sites showed a low dissolved oxygen concentration, depressed pH values and high specific conductivity, commonly associated with acid mine waters. Tar and Miami receives the combined flow of the previous mine discharge sites. Dissolved oxygen concentration is relatively high and the specific conductance elevated. The pH of Tar Creek at this site varied from 3.3 to 7.2, depending on the oxidation of metals and flow from the mine discharge sites.

Water Analysis

The concentration of sodium, magnesium, calcium, potassium and manganese (data not shown) as well as iron, zinc, lead, cadmium, and copper was determined for all sites (Table 2). The calcium and magnesium values were elevated, due to the dissolution of carbonates by the acidity. Zinc concentrations at all sites exceeded the EPA's maximum acceptable concentration (MAC) whereas cadmium exceeded MAC at OWRB #4[15]. Other values for lead, cadmium, and copper fell within the MAC. Iron

concentrations were extremely high, over 400 mg/l, at the mine discharge sites.

Sulfides of iron, zinc, lead and cadmium were the major contaminants dissolved in the surface mine waters. Previous research has indicated that no biologically significant concentrations of other toxic metals, e.g. Se, Hg, Cr etc. were present[16].

Specific conductance values decreased between the time of collection and start of experiment (Table 1). This was attributed to the oxidation of ferrous ion and precipitation of amorphous ferric hydroxide. All changes were monitored by further atomic absorption analysis.

The Effects of the Tar and Treece Samples on Xenopus Survival, Development and Growth

The 96 h EC50 (malformation) and LC50 and the minimum concentrations to inhibit development and growth for all of the samples are presented in Table 3. In all of the Tar and Treece samples there was little or no malformation or death above the level of the controls. Figure 2 is typical of the results of the Tar and Treece samples where no effect was seen in the unadjusted sample from the second sample collection. There was no inhibition of development after 24 h (stages 25-27) by any of the

Tar and Treece samples compared to controls (Table 3). No growth inhibition was seen in either the unadjusted or adjusted samples from the first collection. Growth inhibition was observed in the unadjusted and adjusted samples from the final three collections, by as much as 10% in the highest concentrations. The pH of each concentration at 96 h ranged from 7.4 to 8.1 in both the unadjusted and adjusted samples for the four sample collections (Figure 2).

The Effects of the OWRB #4 Samples on *Xenopus* Survival, Development and Growth

The 96 h LC50 for the first unadjusted OWRB #4 sample was 13.1% concentration and 15.8% concentration for the second sample. The EC50 (malformation) was 13.6% and 13.1% respectively. The ranges of mortality and malformation were the same in each case. In the first adjusted sample 40% of the embryos were dead at 100% concentration and the EC50 was 98.4%. The results from the second adjusted sample along with final pH measurements and metal analysis indicated a pH adjustment problem. Developmental inhibition was observed in the unadjusted samples, where at 24 h control embryos were at stages 25-26 while exposed embryos were at stages 23-25. Growth was inhibited by as much as 47% (compared with controls) in the unadjusted samples

and by as much as 25% in the first adjusted sample (data not shown). The embryos were most sensitive to teratogens during the period of 48 to 96 h exposure, with improper coiling of the gut, pericardial and ventral fin edema, micropthalmia and slight tail kinking being the observed malformations. Death most commonly occurred during the final 48 h of exposure. The pH range for the unadjusted concentrations at 96 h was from 7.4 at 10% to 4.6 at 100% and from 7.5 at 10% to 6.8 at 100% in the first adjusted sample.

The Effects of the Horse Pasture Samples on *Xenopus* Survival, Development and Growth

In all four unadjusted samples the 96 h LC50 was approximately the same as the corresponding EC50 malformation (Table 3 and Figure 3A-D). In the first and fourth adjusted samples the EC50 was somewhat lower than the LC50 (Figure 3A and 3D). The results of the second and third adjusted samples indicated a problem with adjustment to or maintenance of a pH of 7.0. Inhibition of development was observed in all four unadjusted samples and in the fourth adjusted sample (Table 3). Exposed embryos were at stages 23-25 while control embryos were at stages 25-27. Growth inhibition was seen in all samples with inhibition by as much as 53% in the unadjusted sample and 40% in

the adjusted samples (Figure 4). The embryos were most sensitive to death and malformation from 24 to 72 h in the unadjusted samples and from 72 to 96 h in the adjusted samples. Observed malformations were improper coiling of the gut, pericardial and ventral fin edema, micropthalmia and slight to moderate tail kinking. The final pH range was from 7.7 at 10% to 4.3 at 100% for the unadjusted samples (Figure 3A-D) and from 7.7 at 10% to 8.2 at 100% in the first and fourth adjusted samples.

A portion of the Horse Pasture water from the third sampling was passed over Chelex® 100 at pH 7.0 to remove metal ions. Embryos exposed to this water showed no mortality or malformation over control levels.

Effects of the Tar and Miami Samples on *Xenopus* Survival, Development and Growth

In the first and second unadjusted Tar and Miami samples the LC50 was 30 to 40% higher than the corresponding EC50 (malformation) (Table 3). The third unadjusted sample had no effect and the fourth unadjusted sample had an EC50 (malformation) of 90% and no death at 100% concentration. In three of the four adjusted samples 40-50% of the embryos were malformed at 100% concentration. Little or no death occurred in these samples (Figure 5A-D). Developmental inhibition was seen

in the first and second unadjusted samples with exposed embryos at stages 23-25 and control embryos at stages 25-26 at 24 h (Table 3). The third and fourth unadjusted samples and the adjusted samples showed no inhibition of development. Inhibition of growth was observed in all samples with inhibition by as much as 27% in the unadjusted samples and 11% in the adjusted samples (Figure 6). The most sensitive stage for abnormal development in the embryos was from 72 to 96 h. In the first two samples (unadjusted and adjusted) improper coiling of the gut and pericardial and ventral fin edema were the observed malformations. In the fourth collection the malformations in both the unadjusted and adjusted samples were a looser coiling of the gut (as compared to controls) and slight ventral fin edema. In all samples in which malformations were recorded, decreased body pigmentation and swimming ability was also observed. This was true for the Horse Pasture and OWRB #4 samples as well. Death was limited to the first 24 h in the first two unadjusted Tar and Miami samples. The pH values ranged from 7.9 at 10% to 3.2 at 100% in the first two unadjusted samples and from 8.1 at 10% to 7.5 at 100% in the third and fourth samples (Figure 5A-D). The adjusted samples had a final pH range from 8.1 at 10% to 7.4 at 100%.

The Effects of the Tar and Miami Chelated Samples on *Xenopus* Survival, Development and Growth

The Tar and Miami samples caused little or no malformation or death in either the unadjusted or adjusted samples after passage over the Chelex® 100 columns (Figure 7). No developmental inhibition was observed in the chelated samples with all embryos at stages 26-27. The chelated samples also showed no growth inhibition. The average length of the embryos was actually greater than the controls for most of the concentrations of the chelated samples (Figure 8). The final pH values of the unadjusted chelated sample ranged from 7.9 at 10% to 6.8 at 100% and from 8.0 at 10% to 7.0 at 100% in the adjusted sample.

DISCUSSION

Toxicity and Teratogenicity of Heavy Metals

The metal analyses of the Tar Creek samples indicated the presence of several heavy metals in varying amounts (Table 2). Lead, zinc and cadmium have been shown to be teratogenic and toxic in a variety of test animals. Lead was shown to cause teratogenicity and fetal toxicity in rats[17]. Cadmium induced malformations in hamsters although this effect was reversed by the presence of zinc which was weakly teratogenic alone[18]. In chick embryos[19] Cd^{++} was toxic and Cd^{++}, Pb^{++} and Zn^{++} were teratogenic[20]. Cadmium and lead were teratogenic and toxic in Xenopus, with the effects increasing in severity as magnesium concentrations were reduced[21]. For this reason magnesium chloride and calcium chloride were added back to the chelated samples at levels corresponding to those found in our diluent waters. The malformations observed in this study were

similar to those reported by Miller and Landesman[21]. Reduced swimming ability, improper pigmentation and paralysis were also observed.

The Tar and Treece Sample Site

The Tar and Treece site was upstream from the pollution sources. The pH and dissolved oxygen content at this point on the creek were sufficient to support aquatic life. The heavy metal content was lower at this site than the other sites but some metals were present in amounts higher than normally associated with natural freshwaters, most likely due to runoff from adjacent tailings piles. The results indicated that the water at Tar and Treece was not toxic or teratogenic, evidently the metals in the creek were not present in sufficient amounts or speciated in forms most harmful to development. The variability in growth inhibition between the four samples can be explained by the changes in physical parameters, metals and organic content of the creek water between collection times.

The results of the tests on Tar and Treece sample water were significant in that FETAX was able to detect negatives. In this case the Xenopus embryos were able to develop normally in sample water from the portion of the creek that supports life.

The OWRB #4 and Horse Pasture Sample Sites

The OWRB #4 and Horse Pasture sites were surface seeps for mine waters, a source of the metals pollution in the creek. The physical parameters; low pH and dissolved oxygen content and high specific conductivity and heavy metal content were indicative of an environment incompatible with most forms of aquatic life. The test results indicated that both sample waters were extremely toxic with LC50 values at low sample water concentrations. This toxicity was not solely a result of low pH, since the adjusted samples were still toxic. It was important to note that metals precipitated out of solution upon pH adjustment to 7.0. This was significant since a reduction in metal content may have been responsible, in addition to the pH change, for the reduced toxicity in the adjusted samples as compared to the corresponding unadjusted samples. The changes in the proximity of the EC50 (malformation) and LC50 values between the corresponding unadjusted and adjusted samples may also have been an indication of a reduction in toxicity due to reduced metal content as well. When the concentration of metals was lowered, the LC50 values were reduced more than EC50 (malformation) values. This took on added significance when the concentration of metals was reduced, as when the mine water mixed with creek water. Toxicity may be

greatly reduced or eliminated but teratogenic effects may still be exerted. This was seen downstream from the mine sites, at Tar and Miami.

Developmental inhibition occurred when the rate of embryonic development was slowed due to chemical inhibition of metabolism, and was based on the stage of development attained after 24 h. The inhibition of development seen in these samples was attributed to pH and metals. Development was inhibited in the unadjusted samples but not in most of the adjusted samples. However, it could not be assumed that developmental inhibition was only due to pH. The metal content may have been high enough that some metal ions were able to penetrate the protective fertilization envelope and slow development in the first 24 h in the unadjusted samples. Evidence for this conclusion came from the final adjusted sample from the Horse Pasture, in which development was inhibited at as low as 70% concentration of the sample water.

In this same manner, the growth inhibition seen in the OWRB #4 and Horse Pasture samples was possibly due to a combined effect of pH and metals. Variability in metal content and pH were most likely responsible for differences in the degree of inhibition between the samples. Since growth inhibition was

recorded after 96 h of development, metals absorbed by the embryos had sufficient time to exhibit a more profound effect on the embryos than in the first 24 h. Therefore, metals may have been responsible for a greater degree of the growth inhibition than developmental inhibition.

The Tar and Miami Sample Site

The Tar and Miami site was downstream from the area of mine water surfacings and received flow from these seeps as well as flow from the creek above these sites. Freshwater and mine water mix in the creek and effect the water quality of the creek at this site. The great differences in pH and metal content at different collection times were due to the volume of flow into the creek from the mine water surfacings. The low pH of the first two samples was responsible for the mortality that occurred in the highest concentrations of the first two unadjusted samples. The pH was well below 4.0 in the stream samples in which 100% mortality was observed and death took place in the first 24 h. In the third and fourth Tar and Miami samples, with pH values of 7.0 and 6.8, respectively at collection, there was little or no mortality throughout the 96 h test period. This was also true for all the Tar and Miami adjusted samples. The large differences in EC50 (malformation) and LC50 values between the

corresponding unadjusted and adjusted samples, in which an effect was seen, indicated that the water contained teratogens. The mixing of mine water with the creek water reduced the heavy metal concentration and nearly eliminated the toxicity but did not alter the point at which 50% of the embryos were malformed to such a great degree. This can be seen most effectively by comparing the EC50 (malformation) and LC50 values of the mine water surfacing samples and the same values for the Tar and Miami samples (Table 3).

The developmental inhibition seen in the first two unadjusted samples was most likely due to a combined effect of the metals and pH. There was no developmental inhibition in the other two unadjusted samples or any of the adjusted samples in which lower metal content and higher pH values were observed. Growth inhibition occurred in all the Tar and Miami samples including the third sample in which no other effects were seen. Although metal levels were low in this sample, enough metals may have existed in toxic species to inhibit growth.

The Tar and Miami Chelated Samples

The removal of the metal ions in the fourth Tar and Miami sample by passage of the water over Chelex® 100 resulted in removal of the teratogenic and growth inhibiting agents in the

water. Such ions as Cd^{++}, Pb^{++}, and Zn^{++} were bound in the column[10,11] and these metals have been reported to be teratogenic in a variety of both mammalian and non-mammalian test organisms[17-20], including Xenopus[21]. The addition of calcium and magnesium back to the sample after passage over the column ensured that had the embryos been malformed, the malformations would not have been due to the lack of these essential metals.

We also tested one Horse Pasture sample after adjustment of the pH to 7.0 and passage over Chelex® 100. The sample did not cause teratogenicity or toxicity and growth and development were not inhibited.

We concluded, therefore, that heavy metals such as lead, cadmium and zinc in the ionic form were the agents causing the malformations and growth inhibition in the Tar and Miami samples and the mortality, malformations and growth inhibition observed in the mine water samples. The pH may be responsible to some degree for the toxic and growth inhibiting effects and to a greater degree for the developmental inhibition as well. We have not ruled out the possibility that low pH values may increase the severity of the malformations observed. Past indications are

that low pH alone is not teratogenic, but further testing is needed before that conclusion is validated.

It was not a goal of this study to fully understand all of the relationships that existed between pH and metal toxicity. Both pH and metal content changed after dilution in experiments using unadjusted water samples. This made it difficult to determine whether the observed toxicity and teratogenicity were due to pH or metal content. While experiments using the adjusted water samples helped resolve this issue somewhat, additional experiments could be performed that would examine this relationship more fully. For instance, a series of experiments could be conducted where the pH would be held constant in a tolerable pH range and the metal content reduced by dilution. The pH for each dilution series would gradually rise and the effects of a particular pH and its relationship to toxicity and teratogenicity could be observed. Another series of experiments could employ a mixture of reagent grade metal ions that would be combined together based on the metal ion analysis of Tar Creek water. Individual ions could be removed in each series of experiments and the effect of the mixture on toxicity and teratogenicity seen. Additional experiments such as these can be performed using FETAX in a time and cost-effective manner.

The Use of FETAX for Complex Mixtures

The results of this study show that FETAX is excellent for testing the complex mixtures in field samples. The assay not only detected toxicity and determined the reasons for the toxicity, but also showed a teratogenic effect, due to metals, that would not have been determined in an on-site study. The main danger to the embryo was either mortality or growth inhibition at lower concentrations in samples from sites where the mortality and malformation concentration curves overlapped. However, because many embryos were severely malformed, teratogenicity can not be overlooked. The teratogenic risk was greater in samples from sites where the mortality and malformation curves were clearly separate, such as Tar and Miami. By adjusting the physical parameters of the samples the teratogenic, toxic and growth inhibiting effects were resolved and the likely reasons for the effects determined. The goal of an assay is to detect the effects and determine the causes of the effects. This has been accomplished with FETAX.

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Table 1. Temperature, Dissolved Oxygen Content and Specific Conductivity of Tar Creek Samples.

Site:	Tar and Treece				OWRB #4		Horse Pasture				Tar and Miami			
Sampling Date[a]:	5/23	6/24	8/9	9/9	5/23	6/24	5/23	6/24	8/9	9/9	5/23	6/24	8/9	9/9
Temperature (°C)														
Collection site	24.0	25.0	NS[b]	27.0	19.0	18.0	20.0	18.0	NS	19.0	25.0	26.0	NS	26.0
Dissolved oxygen (mg/l)														
Collection site	8.0	7.2	NS	6.6	2.9	1.5	2.4	1.5	NS	1.0	10.0	7.5	NS	8.7
Experiment @start	6.6	7.2	7.4	8.4	2.2	2.8	1.7	1.5	1.4	1.1	8.2	7.7	8.2	7.5
Experiment @48 hr	9.0	10.0	7.7	8.7	4.2	4.0	3.9	2.7	2.7	2.4	10.1	11.2	8.9	8.1
pH (S.U.)														
Collection site	7.4	7.0	7.3	7.3	5.5	5.6	6.0	6.0	6.4	6.0	3.3	3.3	7.2	6.8
Experiment @start	7.3	7.4	7.5	7.3	5.4	5.4	5.9	5.8	5.7	5.8	3.4	3.2	7.2	6.4
Experiment @48 hr	7.2	7.4	7.4	7.4	5.4	5.4	5.9	5.8	5.8	5.9	3.3	3.1	7.2	6.3
Specific Conductivity (μMHOS/cm)														
Collection site	1040	1250	2100	1680	3280	3220	3320	3390	3500	3360	2180	2650	2200	1380
Experiment @start	380	570	90	1190	1500	1580	1480	1670	1740	1750	870	1080	930	990
Experiment @48 hr	350	470	710	1050	1400	1310	1370	1500	1620	1590	730	830	740	870

[a] Date sample was collected.

[b] Not sampled.

Table 2. Concentration Ranges of Divalent Metal Ions for Each Sample Site[a].

Sample Site	Iron (mg/l)	Zinc (mg/l)	Lead[b] (mg/l)	Cadmium[b] (mg/l)	Copper (mg/l)
Tar and Treece	<0.04-5.59	2.20-5.86	<0.005-0.006	<0.005-0.027	<0.04
OWRB #4	292.0-433.0	178.0-235.0	<0.005-0.008	0.055-0.104	<0.04
Horse Pasture	414.0-533.0	47.2 73.0	<0.005-0.006	<0.005-0.006	<0.04-0.04
Tar and Miami	10.0-90.8	7.5 78.0	<0.005-0.008	<0.005-0.043	<0.04
Tar and Miami[c]	0.11	<0.1	0.006	0.006	<0.04

[a] Single determinations for each sampling date during 1983-84 make up the range of values presented.

[b] Samples analyzed by graphite furnace (HGA) and L'vov platform. Matrix interferences may be suppressing detection of these elements in the mine water discharge and the Tar and Miami sites.

[c] Water sample passed twice over Chelex® 100 column.

Table 3. Effect of Tar Creek Water on Malformation, Mortality, Rate of Development and Growth of Xenopus Embryos.

Sample Site	96 h EC50[a] malformation (% conc)	96 H LC50[a] mortality (% conc)	Min. conc. to inhibit develop.[b] (% conc)	Min. conc. to inhibit growth[c] (% conc)
Tar and Treece				
pH unadjusted				
5/30/84-pH 7.3[d]	0/100	0/100	DNI	GNI
7/10/84-pH 7.4	5/100	0/100	DNI	40
8/14/84-pH 7.5	5/100	0/100	DNI	60
9/19/84-pH 7.3	0/100	0/100	DNI	90
pH adjusted				
5/30/84-pH 7.0	0/100	5/100	DNI	GNI
7/10/84-pH 7.0	5/100	0/100	DNI	80
8/14/84-pH 7.0	10/100	0/100	DNI	70
9/19/84-pH 7.0	10/100	0/100	DNI	90
OWRB#4				
pH unadjusted				
5/30/84-pH 5.4	13.6	13.1	50	30
7/10/84-pH 5.4	13.1	15.8	30	10
pH adjusted				
5/30/84-pH 7.0	98.4	40/100	DNI	50
7/19/84-pH 7.0	16/100	5/100	DNI	30

Table 3. cont.

Horse Pasture				
pH unadjusted				
5/30/84-pH 5.9	35.4	31.0	40	30
7/3/84-pH 5.8	34.0	28.4	40	10
8/21/84-pH 5.7	53.3	54.3	50	40
9/25/84-pH 5.8	30.3	27.7	30	20
pH adjusted				
5/30/84-pH 7.0	79.7	99.0	DNI	80
7/3/84-pH 7.0	15/90[e]	0/90[e]	DNI	60
8/21/84-pH 7.0	0/100	5/100	DNI	100
9/25/84-pH 7.0	70.4	40/100	70	20
Tar and Miami				
pH unadjusted				
5/30/84-pH 3.4	44.7	84.4	40	40
7/10/84-pH 3.2	40.1	74.8	40	30
8/14/84-pH 7.2	0/100	0/100	DNI	60
9/11/84-pH 6.4	90.0	0/100	DNI	70
pH adjusted				
5/30/84-pH 7.0	40/100	0/100	DNI	90
7/10/84-pH 7.0	49/100	7.5/100	DNI	60
8/14/84-pH 7.0	0/100	0/100	DNI	60
9/11/84-pH 7.0	40/100	0/100	DNI	70

Table 3. cont.

Tar and Miami Chelated[f]				
pH unadjusted				
9/18/84-pH 6.4	0/100	7.5/100	DNI	GNI
pH adjusted				
9/18/84-pH 7.0	0/100	0/100	DNI	GNI

[a] The 0/100 format indicates a 0% response at 100% concentration. This format was used when a 50% response was not attained.

[b] Based on stage of development at 24 h. DNI - Development not inhibited after the first 24 h of test.

[c] GNI - Growth not inhibited.

[d] The date listed is the FETAX start date and the water pH on that date.

[e] The response at 90% concentration is listed since all embryos at 100% concentration died within the first 24 h due to a pH adjustment problem.

[f] Tar and Miami sample water was passed over a Chelex® 100 column to remove divalent cations.

FIGURE LEGENDS

Figure 1. Tar Creek area map and locations of study sites.

Tar and Treece-Tar Creek and Treece, OK	S18 T29N R23E
OWRB# 4-East of Tar and Lytle Creek junction	S29 T29N R23E
Horse Pasture-South of Commerce, OK	S7 T28N R23E
Tar and Miami-N.E.O. A&M and Tar Creek bridge	S30 T28N R23E

Figure 2. The effect of increasing concentrations of Tar and Treece water on Xenopus malformation (□) and mortality (△) after 96 h. Tar and Treece Sample #2 (7/10/84) was diluted with dechlorinated tap water. The pH (○) of each concentration was then measured at 96 h.

Figure 3. The effect of increasing concentrations of Horse Pasture water on Xenopus malformation (□) and mortality (△) after 96 h with no adjustment of pH. Horse Pasture

samples were diluted with dechlorinated tap water. The pH (○) of each concentration was then measured at 96 h. Malformation (■) and mortality (▲) due to pH adjusted samples. Panel A-sample 5/30/84, panel B-sample 7/3/84, panel C-sample 8/21/84, panel D-sample 9/25/84.

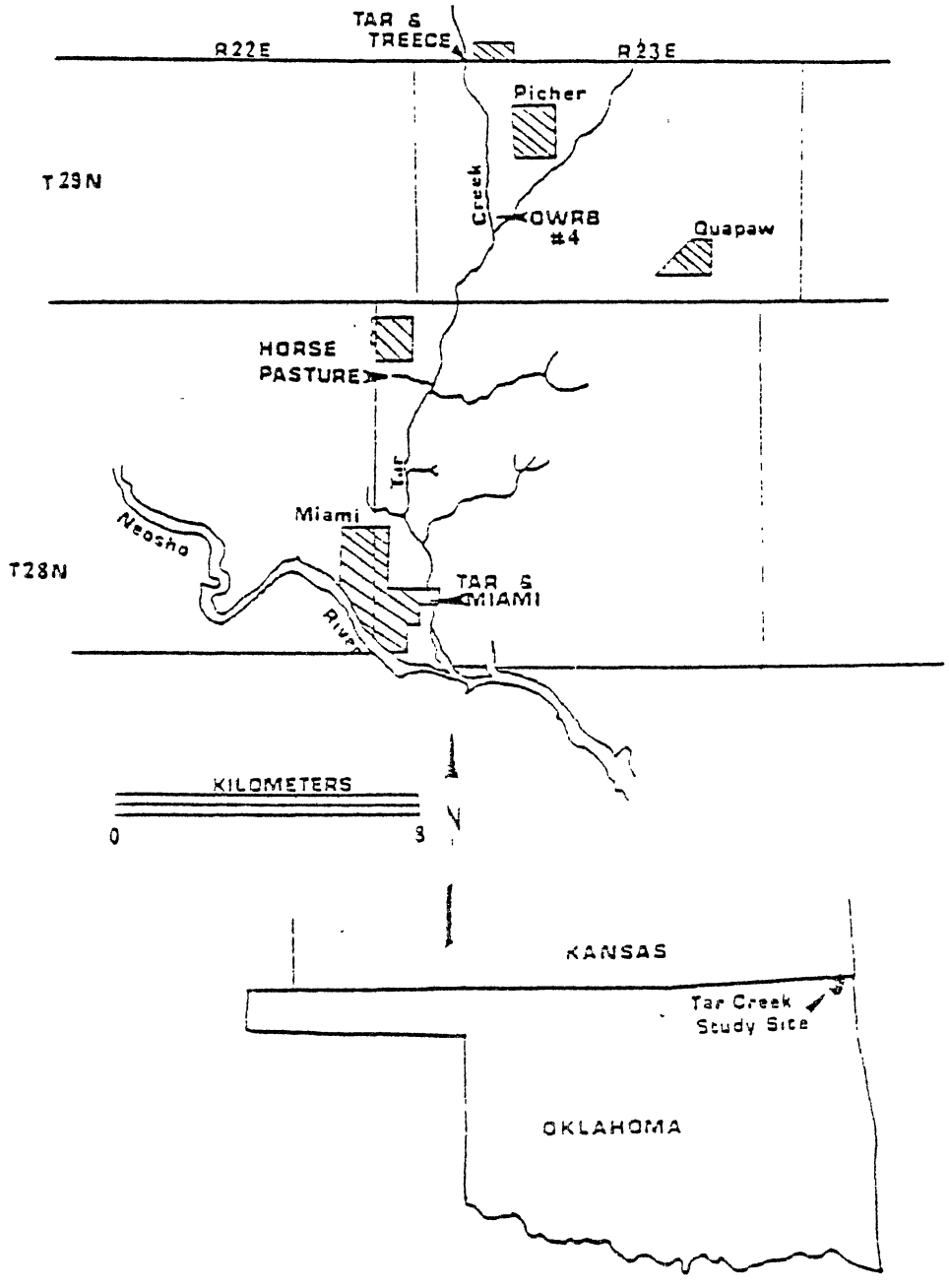
Figure 4. The effect of increasing concentrations of Horse Pasture water on the inhibition of Xenopus growth (▽) after 96 h with no adjustment of pH. Horse Pasture samples were diluted with dechlorinated tap water. Growth inhibition (▼) due to pH adjusted samples. Panel A-sample 5/30/84, panel B-sample 7/3/84, panel C-sample 8/21/84, panel D-sample 9/25/84.

Figure 5. The effect of increasing concentrations of Tar and Miami water on Xenopus malformation (□) and mortality (△) after 96 h with no adjustment of pH. Tar and Miami samples were diluted with dechlorinated tap water. The pH (○) of each concentration was then measured at 96 h. Malformation (■) and mortality (▲) of pH adjusted samples. Panel A-sample 5/30/84, panel B-sample 7/10/84, panel C-sample 8/14/84, panel D-sample 9/11/84.

Figure 6. The effect of increasing concentrations of Tar and Miami water on the inhibition of Xenopus growth (▽) after 96 h with no adjustment of pH. Tar and Miami samples were diluted with dechlorinated tap water. Growth inhibition (▼) due to pH adjusted samples. Panel A-sample 5/30/84, panel B-sample 7/10/84, panel C-sample 8/14/84, panel D-sample 9/11/84.

Figure 7. The effect of increasing concentrations of Chelex® 100-treated Tar and Miami water on Xenopus malformation and mortality after 96 h. Panel A-malformation (□) and mortality (△) with no Chelex® 100-treatment or adjustment of pH. Panel B-malformation (□) and mortality (△) due to pH unadjusted sample after passage over Chelex® 100. Sample water collected 9/11/84.

Figure 8. The effect of increasing concentrations of Chelex® 100-treated Tar and Miami water on the inhibition of Xenopus growth after 96 h. Inhibition of growth (▽) due to pH unadjusted sample water. Inhibition of growth (▼) due to pH unadjusted sample water after passage over Chelex® 100. Sample water collected 9/11/84.


TAR &
TREECE
R22E
R23E
Picher
T29N
Creek
OWRB
#4
Quapaw
HORSE
PASTURE
Tar
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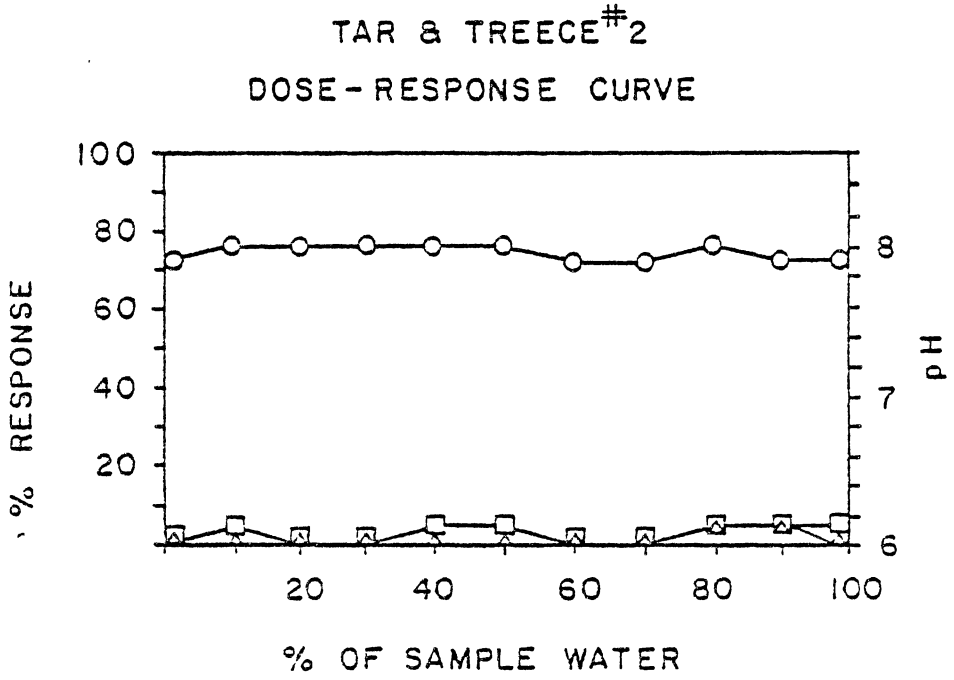
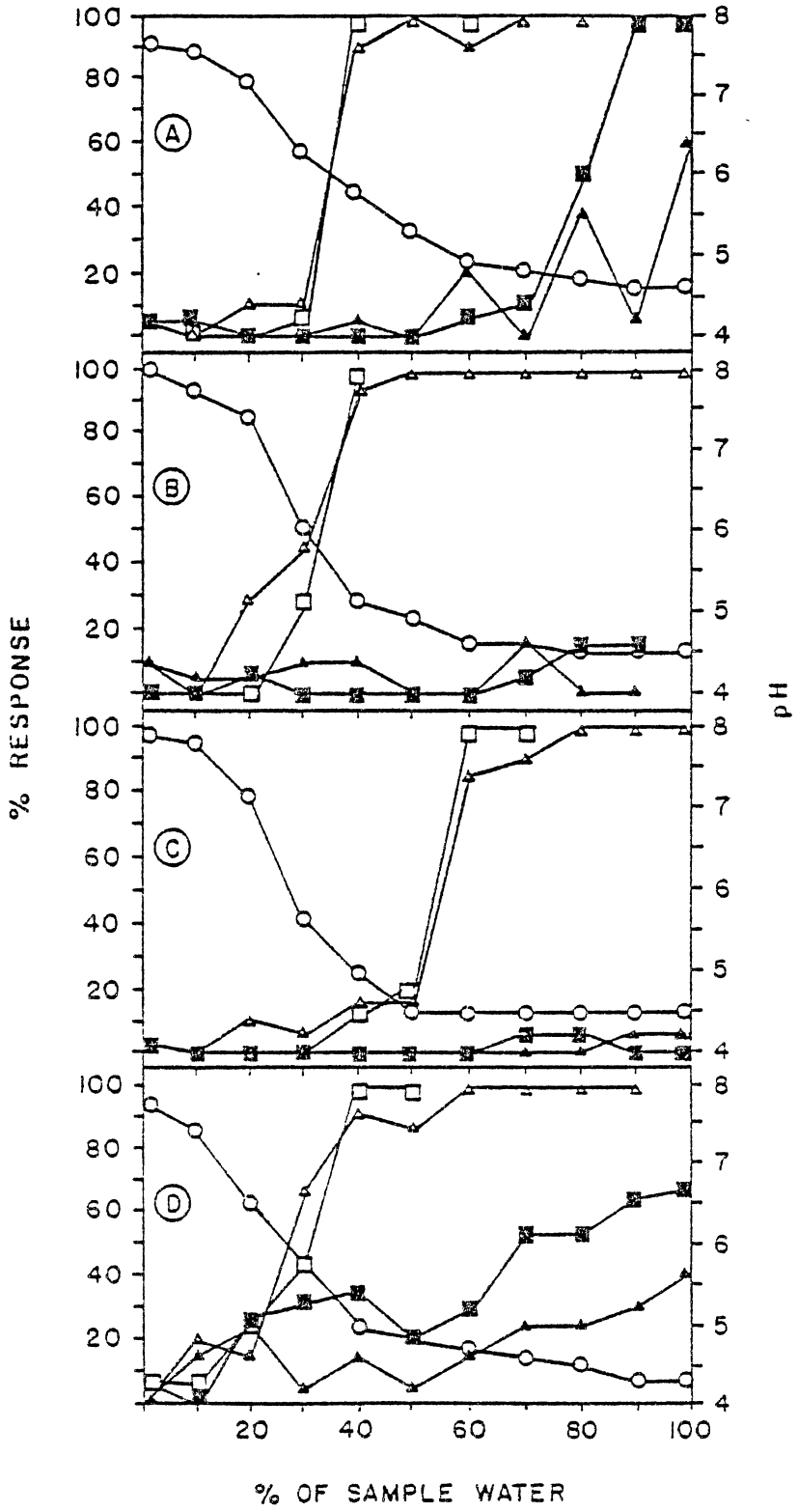
Neosho
T28N
TAR &
MIAMI
River
KILOMETERS
0
3
KANSAS
Tar Creek
Study Site
OKLAHOMA


TAR & TREECE#2
DOSE-RESPONSE CURVE
100
80
60
40
20
% RESPONSE
8
7
6
pH
20
40
60
80
100
% OF SAMPLE WATER

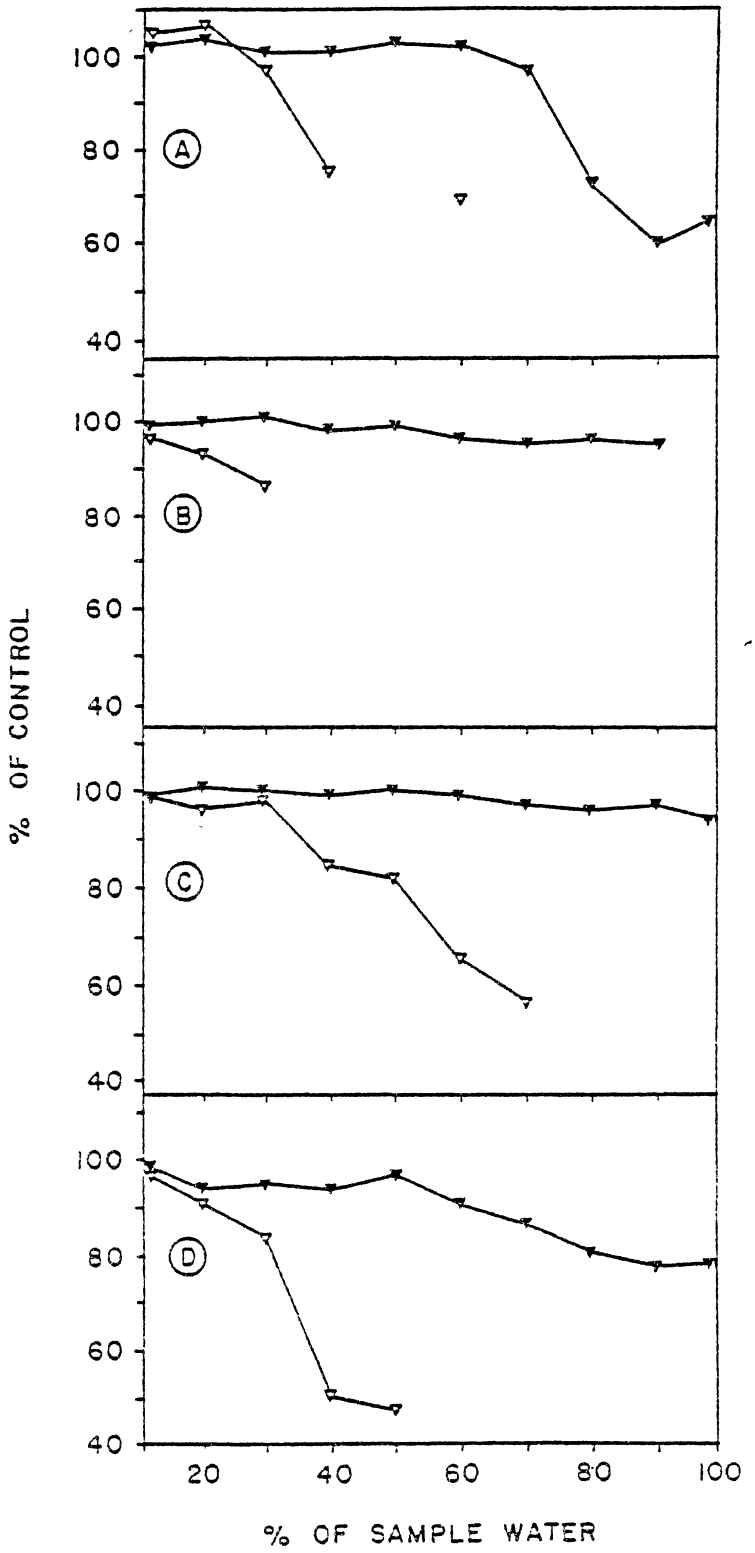
HORSE PASTURE

DOSE - RESPONSE CURVE



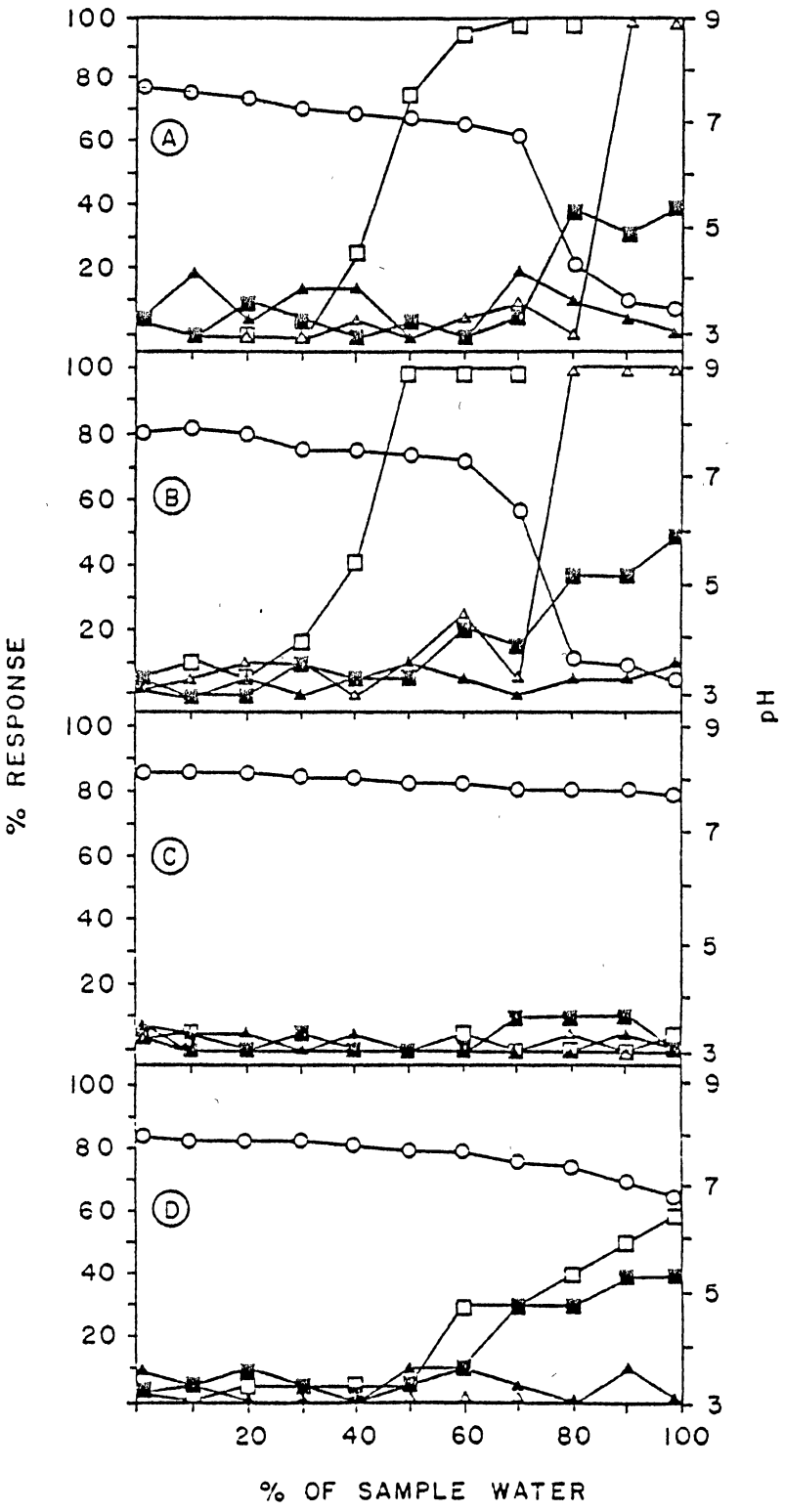
HORSE PASTURE

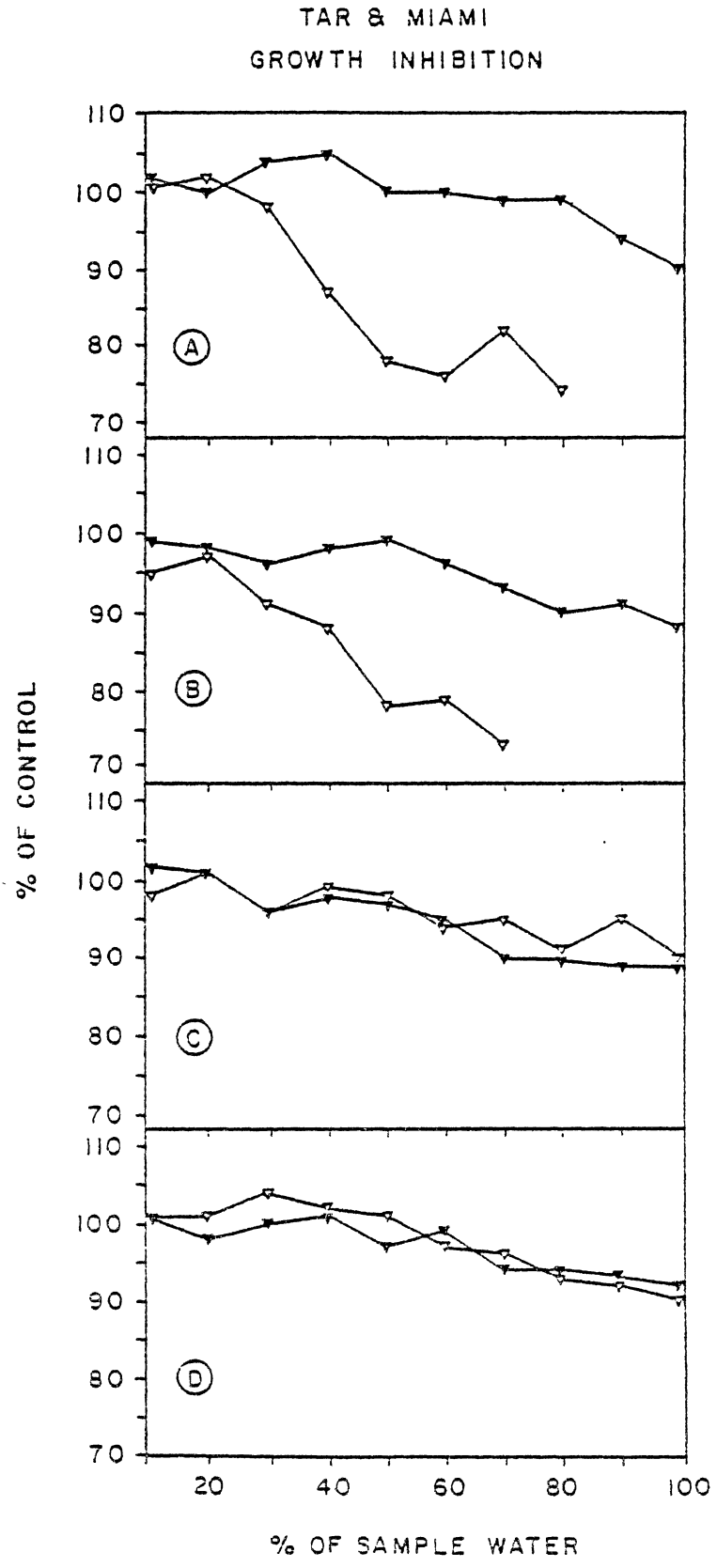
GROWTH INHIBITION



TAR & MIAMI

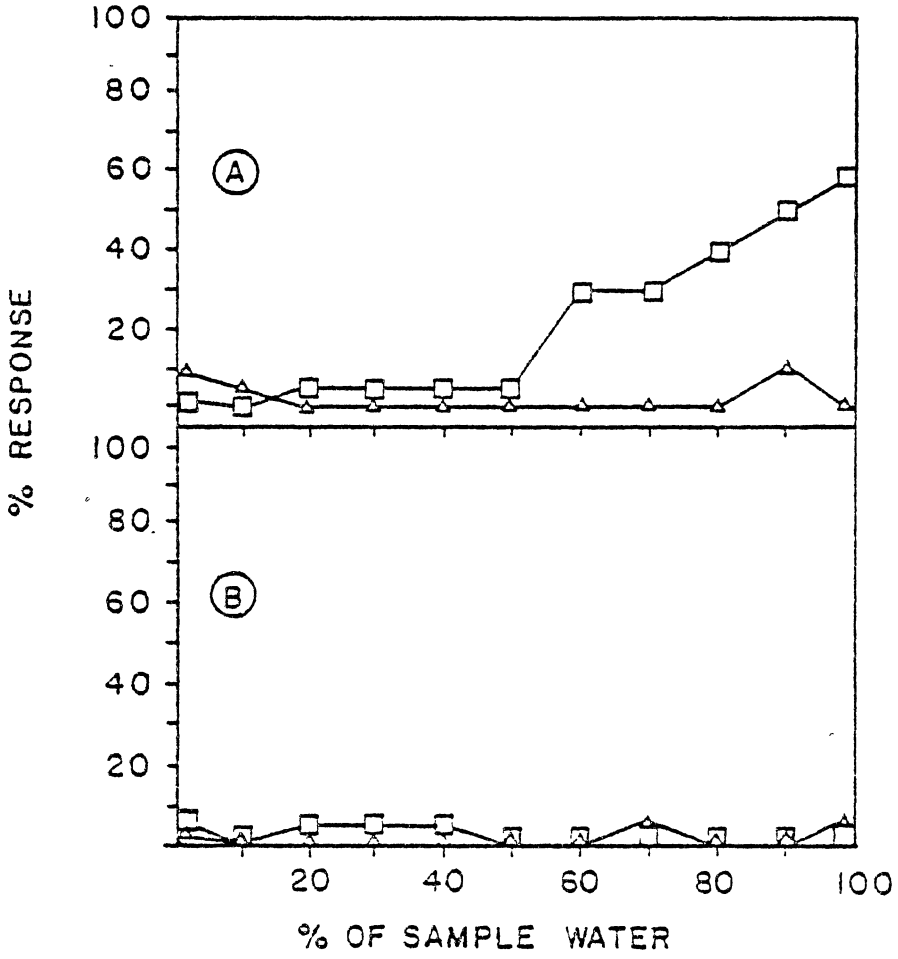
DOSE - RESPONSE CURVE

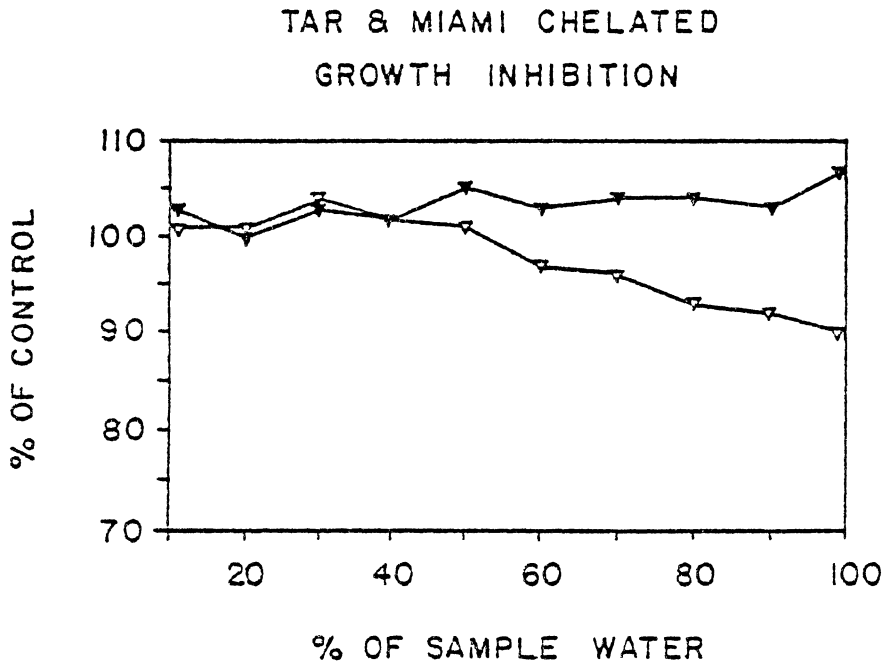



TAR & MIAMI
GROWTH INHIBITION
% OF CONTROL
110
100
90
80
70
A
B
C
D
20
40
60
80
100
% OF SAMPLE WATER

TAR & MIAMI CHELATED

DOSE-RESPONSE CURVE




TAR & MIAMI CHELATED
GROWTH INHIBITION
110
100
90
80
70
% OF CONTROL
20
40
60
80
100
% OF SAMPLE WATER

VITA 2

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Candidate for the Degree of

Master of Science

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